How Current Direct-Acting Antiviral and Novel Cell Culture Systems for HCV are Shaping Therapy and Molecular Diagnosis of Chronic HCV Infection

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Abstract: We have entered a new era of hepatitis C virus (HCV) therapy in which elimination of infection and disease is a real possibility. HCV cell culture models were instrumental for identification of therapeutic targets, testing candidate drugs, and profiling of therapeutic strategies. Here we describe current and novel methods of cell culture systems for HCV that are allowing investigation of HCV life cycle and virus-host interaction required for replication and propagation. The development of protocols to grow infectious virus in culture and generate hepatocyte cell lines from specific individuals hold great promise to investigate the mechanisms exploited by the virus to spread the infection and the host factors critical for HCV replication and propagation, or resistance to infection. Since host factors are assumed to be conserved among HCV isolates and genotypes and antagonizing molecules should possess high genetic barrier to viral resistance, the development of drugs targeting host factors essential for virus replication holds great promises in further increasing treatment efficacy. Refocusing of therapeutic goals also impacted *in vitro* diagnosis. The primary goal of anti-HCV therapy is to achieve a sustained virologic response (SVR) defined as " undetectable" HCV RNA genome in the serum or plasma at 12 to 24 weeks following the end of treatment. Use of direct antiviral agents has substantially changed threshold of the viral load used to define SVR and led to a reassessment, discussed herein, of result interpretation and requirements of clinically-approved, quantitative molecular assays.

Keywords: Cell culture-derived HCV; Hepatitis C virus; HCV cell culture systems; Induced pluripotent stem cells; Molecular diagnosis; Pseudoparticles; Quantitative molecular assay; Replicon.

1. INTRODUCTION

As clearly evident from previous chapters of this issue, we are witnessing an epochal change in the management and therapy of HCV infection. Up to four years ago there were drug regimens modestly effective, which required prolonged treatment and caused significant side effects. This gloomy picture dramatically changed with the advent of novel molecular techniques and computer modeling, and the development of *in vitro* systems to study replication, pathogenetic mechanisms, and, ultimately, Achilles' heel of HCV life cycle. Intense use of these technologies had its payoff to the point that some obscure points of viral replication and interaction with cell and host have been unveiled [1] and novel antiviral molecules were developed. These directly target the virus and, in preliminary clinical tests, proved easy-to-administer, well tolerated, and surprisingly effective. These features and, by contrast, poor performances of conventional therapies ensured fast lane for clinical approval to several drugs.

Novel direct antiviral agents (DAAs) completely changed scenario and expectance of patients: some DAAs, either alone or in combination with interferon (IFN) and ribavirin (RBV) - the mainstay of therapy until a few years ago - boast cure rates exceeding 95% [2, 3], reduce liver damage even in long-term infected patients, and have high genetic barriers, i.e. the virus can become resistant but at a great cost in terms of reduction of fitness and virulence [4].

Here, we describe current and novel technologies being used to study HCV life cycle, investigate viral-host interaction, and develop highly potent HCV antivirals. These novel drugs changed expectations and management of patients, and, as discussed, caused reassessment of molecular diagnosis of infection and interpretation of results.

2. HCV REPLICATION IN HEPATOCYTES AND PATHWAYS TO DISSEMINATION INTO OTHER TARGET CELLS

HCV was the first human pathogen to be discovered by molecular methods and up to few years ago most of which was known was, indeed, molecular. Since its discovery, occurred in late eighties [5], we clearly understood that the virus has a high degree of genetic heterogeneity [4], which helps the virus to resist immunological and other host pressures, adapt to replicate in various body sites and tissues, and causing, ultimately, rapid inter- and intra-host evolution of the viral genome [6]. This wealth of information contrasted with poor knowledge about virus replication and persistence in infected cells.

The lack of solid cell culture systems in which to replicate and produce infectious HCV virions has represented a major hurdle to study this pathogen and develop strategies for prophylactic and therapeutic interventions. First attempts to adapt patient-derived HCV isolates failed to establish productive infection in cell culture and, as described below, there were no progresses in this direction until 1997, when molecular clones infectious in chimpanzees were developed. Unfortunately these viral genomes were able to generate infectious particles *in vitro* under certain conditions but were unpractical to study key steps of viral replication and antiviral potency of drug candidates [7, 8]. These clones, however, were important landmarks as they paved the way to various surrogate systems, described in the next paragraph and that, together with *in vivo* studies in chimpanzees and recently developed small animal models, greatly contributed to shed light on life cycle, virus–host interactions that mediate infection, establishment of chronicity, and progression of HCV-mediated disease.

HCV exhibits several peculiar features that are still puzzling researchers and hampered the development of drugs directly targeting the virus. As shown in Figure 1, HCV circulates in the bloodstream as lipoviral particle (LPV), in which the virion is surrounded and partly shielded to most immune effectors [9]. Once it reaches its primary target organ, the liver, HCV crosses the fenestrated endothelium and interacts with an ever-increasing complex network of putative receptors which roles and importance in driving virus entry is still uncertain. According to recent molecular models, viral absorption to cell surface is initiated by binding to heparan sulfate proteoglycans (HSPGs) and receptors such as the tetraspanin CD81 on the basolateral side of hepatocytes [10, 11]. The virus then, presumably facilitated by its circulation as LPV, absorbs to two lipoprotein molecules: the scavenger receptor class B, type I (SR-BI) [12] and the low-density lipoprotein receptor (LDLR) [13]. This list of putative receptors increased of six other novel entities in the last eight years: the tight junction molecules claudin-1 (CLDN1) [14] and occluding (OCLN) [15], the epidermal growth factor receptor (EGFR) and the ephrin type-A receptor 2 (EphA2) [16], the cholesterol uptake molecule Niemann-Pick C1-like 1 (NPC1L1) [17], and the transferrin receptor 1 (TFR1) [18]. Since these molecules segregate into different subcellular domains, it is likely that interaction with receptors occurs in a step-wise fashion that actively transports the virus towards the apical side of the hepatocyte where the virus eventually enters by fusion and is internalized by clathrin-mediated endocytosis. Low pH of the endocytic compartment then disassemble the viral capsid that releases the positive single-strand HCV RNA genome that is immediately translated by the host protein synthesis machinery to produce a single HCV polyprotein precursor of about 3000 amino acids [19, 20]. The polyprotein

is then processed by both cellular and viral proteases to give rise to 10 viral proteins: three are structural components of the virion - core, which forms the nucleocapsid, and the viral envelope glycoproteins, E1 and E2 -, and seven are non-structural (NS) proteins that have various functions [21, 22]. A few NS proteins organize in a replication complex that produces multiple copies of the HCV RNA genome via a minus-strand replicative intermediate [23]. Others cleave and mature viral proteins from the polyprotein precursor and help assembly of nucleocapsid to bud into the endoplasmic reticulum from which it eventually acquires the envelope and E1 and E2 glycoproteins. Along the secretory pathway, virions undergo maturation, mainly operated by HCV protease, and associate with endogenous lipoproteins to form LPVs [9, 24]. LPVs are eventually released from the cell to start a new round of infection.

In addition to dissemination and infection via the bloodstream, HCV actively exploits the so called "cell-to-cell" spread [25, 26]. Here, the virus released by an infected cell directly infects neighboring cells without diffusion through the extra-cellular environment. Cell-to-cell transmission occurs through the engagement of two co-receptors, CLDN1 and OCLN, at the intercellular interface (Figure 1). Compared to "cell-free" spread, cell-to-cell transmission is thought to be an important mechanism of HCV persistence as it provides several advantages as regards efficiency of infection, viral dissemination, immunological escape, and resistance to DAAs [25, 27-31].

Even though hepatocytes are the main site for viral replication, a broad clinical spectrum of extrahepatic complications and diseases are associated with chronic HCV infection, which suggests the existence of some other target cells for viral replication. As described in this thematic issue, B-cell abnormalities and lymphoproliferative disorders

are frequently observed in HCV infected patients [32-35], and, therefore, B-lymphocytes as well as peripheral blood circulating cells are thought to be target of HCV replication. Whereas there are many *in vitro* and *in vivo* studies supporting this hypothesis [36-42], it is unclear what are the molecular pathways used by the virus to enter and replicate in extrahepatic cells. None of these cells expresses all receptors required for viral entry in hepatocytes. Since cell-to-cell transmission appears to be less dependent on virus-host receptor interaction compared to cell-free transmission, it has been postulated that dissemination to extrahepatic cells occurs via cell contiguity or that other viral proteins, beside HCV envelope glycoprotein E2, interact with the target cells and anchor the virus on the cell surface [28, 43, 44]. Evidences supporting cell-to-cell transmission are the close anatomical vicinity of hepatocytes and lymphoid cells in the liver (especially during chronic inflammation) and the presence of CD81 and SR-BI on the lymphoid cell membrane [45, 46]. This finding is particularly relevant since among all above mentioned putative receptors and coreceptors, CD81 and SR-BI are the only two molecules demonstrated to bind the glycoprotein E2 [11, 12]. Evidences against this hypothesis are the absence of CLDN1 and OCLN in lymphoid cells [47] and conflicting results with *in vitro* models [48]. An alternative hypothesis is that HCV infects lymphoid and other non-hepatic target cells using different receptor [28, 47, 49, 50]. Downstream viral entry, it is very likely that HCV interacts with proteins that are absent in hepatocytes and that cells themselves react to infection in different ways, implying once again the necessity for the virus to adapt to the new environmental conditions. Here again there are many studies *in vitro* and *in vivo* corroborating these considerations and ranging from genome evolution following replication in lymphoid cells [39, 51, 52], engagement of specific molecular

pathways [28, 53, 54], different interferon production, cell responsiveness and consequence to infection [34, 55]. Whereas there are many features that need to be uncovered to understand the pathobiology of HCV replication and role of extrahepatic sites in fueling the HCV syndrome [33, 56], non-hepatic cells are clearly important sites of HCV persistence with a relevant impact to antiviral treatment and possibility to eradicate the infection [57, 58].

3. IN VITRO METHODS TO STUDY HCV LIFE CYCLE AND DEVELOP ANTIVIRAL DRUGS

Complicated interactions with putative cellular receptors, association of the virion with endogenous lipoproteins, difficulties to propagate hepatocytes *in vitro*, and adapt the virus to grow in non-hepatic cells, have insofar impeded the development of cell culture systems in which the virus consistently establishes a truly productive infection.

3.1. Conventional *in vitro* methods

As shown in Figure 2, there are four standard methods currently used to study the replication cycle and develop DDAs: 1. The HCV subgenomic replicon; 2. The HCV pseudoparticles (HCVpp); 3. Cell culture derived HCV (HCVcc); 4. HCV trans-complemented particles (HCV_{TCP}).

3.1.1. HCV subgenomic replicon

This system was described in 1999 and is based on a bicistronic RNA construct expressing the minimal set of HCV proteins required to initiate and maintain HCV replication in human cells [59]. The first cistron encoded *neo*, a selection marker placed under control for expression of the HCV internal ribosome entry site (IRES). The second cistron, placed under control of an IRES from a picornavirus, contained the HCV NS3-NS5B segment (Figure 2a). NS3-NS5B encodes the HCV proteins essential for replication, namely: NS3/4A protease, NS4B participating in the replicase complex [60], the NS5A protein, also an essential component of the replicase complex and exerting a wide range of effects on cellular pathways and processes [61], and the RNA-dependent RNA polymerase NS5B. The subgenomic construct was then used as template to transcribe, *in vitro*, an RNA that was transfected in Huh-7 cells – a human continuous cell line of well differentiated hepatocytes derived a hepatocellular carcinoma – or equivalent cells. Cells were finally cultivated in the presence of G418, an antibiotic blocking the polypeptide synthesis in both prokaryotic and eukaryotic cells and inactivated by the *neo* coding protein. Transfection and antibiotic selection resulted in the generation of cell lines stably reproducing some specific steps of viral life cycle. Unfortunately, due to the lack of genes encoding for the structural proteins there was no production of infectious particles (Figure 2a) [59]. This method was scarcely efficient and viral "replication" occurred at low levels, most of the times unpractical for screening of large number of candidate antiviral molecules. To obviate this limitation, the subgenomic replicon underwent several modifications, mainly site-specific mutations that were either naturally selected during the replication process or manually inserted into the subgenomic replicon to produce viral variants with higher replication capability [62-70].

Of note, most of naturally occurring mutations were found localized in the N-terminus of the NS3 helicase [66, 71], NS4B [72] and NS5A [66, 73, 74], thus showing us the protein domains critical for *in vitro* replication and where to intervene for functional adjustments. From the first subgenomic replicon produced, derived from a HCV isolate of genotype 1b, others were described deriving from either other isolates of genotype 1b or isolates belonging to the 6 major genotypes described so far [65, 75-84]). More recently, modified versions have been described, these include the presence of reporter genes such as luciferase (Luc) or green fluorescent protein (GFP) that simplify measurement of replication activity [66, 85], and a tricistronic version also encoding for a reporter gene that was placed under control of an own and independent HCV IRES [86].

3.1.2. HCV pseudoparticles (HCVpp)

Together with a soluble form of the HCV glycoprotein E2 [87], not described here for brevity, the HCVpp model was extensively used to identify viral receptors, study the mechanism of entry, examine host immune response and, more recently, investigate for potential entry inhibitors. HCVpp represented a great advance in HCV research and was inspired by previous works with HIV and viral vectors. Similarly to lentiviral and retroviral vectors, HCVpp was produced from defective retroviral particles that were assembled in such a way to incorporate and express on their surface the HCV envelope glycoproteins [88, 89]. This procedure, called pseudotypization, is extensively used in viral vector production and is exploited, in essence, to alter host tropism of parental virus and target specific tissues, increase/decrease stability of the virus particle, investigate for viral receptors and antibody-mediated neutralization for those viruses for which an easy

and reliable cultivation system is not at hand. Since pseudotyped particles do not carry the structural genes, i.e. genes encoding capsid and foreign viral envelope glycoproteins, they do not generate progeny viral particles [90]. HCVpp are generated by co-transfection of packaging cells, i.e. human cell lines, usually the epithelial 293T cells, easy-to-transfect and expressing the heterologous genes at high levels. Transfection is performed with three plasmids encoding the HCV glycoproteins E1 and E2, the capsid and enzyme proteins of HIV or murine leukemia virus (MLV) necessary for assembly and processing the viral nucleic acid, and a defective HIV or MLV genome containing Luc or GFP as reporter gene. Following transfection, the packaging cells start to produce and release into the supernatant the vector particles that are harvested from the culture and used to transduce the target cells. HCVpp entry into the target cells is determined by measuring Luc or GFP activity one-two days after transduction. Since Luc and GFP signals increase almost proportionally to the number of transduced cells, quantitative evaluation of reporter genes permits precise measurement of HCV entry into target cells (Figure 2b). HCVpp was extensively used to study the role of E1 and E2 glycoproteins in HCV entry, to identify HCV receptors and other factors contributing to cell absorption and to shed light on the mechanisms of virus internalization [91, 92]). This system was also instrumental to elucidate protective role, kinetic of appearance, affinity, and breadth of neutralizing antibodies elicited in infected individuals or immunized animals [93]. In fact, HCVpp became the reference method to analyze the humoral response in acutely and chronically infected individuals. These studies demonstrated that neutralizing antibodies exert marginal role in controlling chronic infection and disease progression [27, 94]. Nevertheless, patients who promptly developed neutralizing antibodies in the

early stage of infection were more likely to eradicate the infection [95, 96]. Finally, HCVpp also played major role to assist in the design of anti-HCV vaccines [97]. Unfortunately, this model has some limitations. First of all, pseudotyped virions are more susceptible to antibody-mediated neutralization compared to naïve isolates; this is likely due to suboptimal organization and/or localization of envelope glycoproteins [98]. Furthermore, at a difference with HCV virions produce in hepatocytes, the HCVpp generated in epithelial HEK-293T cells are not associated with lipoproteins (Figure 2b) and may therefore exhibit different properties concerning interaction with putative receptors and susceptibility to antibody-mediated neutralization [99, 100].

3.1.3. Cell culture derived HCV (HCVcc)

The above two methods neither yield infectious virus nor establish a truly productive infection, which is essential to studying late stages of viral replication, e.g. virion assembly, maturation and release. Further, a productive cell culture would simplify definition and assessment of therapeutic targets. The first experiments to obviate to these limitations were based on adaptation of patient-derived isolates to *in vitro* cell cultures. Unfortunately, and despite countless attempts, this approach was unsuccessful and demonstrated that HCV, like HIV and other patient-isolated viruses, requires adaptation of some molecular and phenotypical properties to *in vitro* culture conditions [101]. Exception to this rule was JFH1, a HCV strain of genotype 2a isolated from a Japanese patient with fulminant HCV-associated hepatitis and found able to replicate at low-levels in Huh-7 (Figure 2c) [102, 103]. The HCVcc system derived from JFH1 was extensively used to study HCV life cycle [104-106], identify novel entry factors, e.g. NPC1L1, EGFR

and EphA2 [16, 17], define morphological and biochemical features of the virion [107-109], and characterize virion association to lipoproteins [110]. Unfortunately no HCVcc systems were available for the clinically more relevant genotypes 1, 3 and 4.

With the idea to establish HCVcc from various genotypes and with higher replication capacity, chimeric viruses were produced using JFH1 as backbone in which were inserted the structural protein coding regions of other isolates. After first successful attempt to produce a chimeric clone from J6 strain (also of genotype 2a) [111] replication-competent chimeric clones were generated from genotype 1 through 7 [112]. Interestingly, propagation of these chimeric clones resulted in the accumulation of scattered mutations, mainly in NS2-NS3 coding region. Some of these were deleterious [104, 113], others progressively adapted the chimeric viruses to *in vitro* conditions and, particularly, hepatoma cells [114-116].

HCVcc systems using genotype 1 subgenomic replicons were extensively used to test efficacy of most DDAs. These HCVcc systems were also used as platform to develop intergenotypic chimeras recombinant in DDA target regions (e.g. NS2, NS3/4a, NS5a and NS5b alone or in combination) were used to assess the antiviral activity against other genotypes. To this purpose and to permit simple and dynamic measurements of viral replication, reporter genes were incorporated into the molecular clones as either fused proteins [117] or additional proteins that were cleaved off by the HCV protease during processing of the polyprotein precursor [118-120]).

JFH1 and HCVcc have been one of the biggest achievements in HCV research. This system opened the way to the development of other full-length, replication-competent molecular clones of genotype 1 and 2 [121, 122]. Nonetheless, we still lack

HCVcc systems for complete genotypes 3, 4, 5 and 6 and only a few isolates of genotype 1 and 2 proliferate in culture. Thus a broad and patient isolate-specific analysis of particular life cycle steps is currently not possible.

3.1.4. HCV trans-complemented particles (HCV_{TCP})

An alternative system to study the whole life cycle of HCV is provided by the trans-complemented JFH1 particles (HCV_{TCP}) and derives from the observation that HCV subgenomic replicons can be packaged when structural proteins are supplied in *trans* [123, 124]. HCV_{TCP} are infectious, relatively easy to produce, thus overcoming the hurdle of isolate specificity, but support only single-round infection and are unable to spread [125]. HCV_{TCP} is generated by cotransfection of a JFH1 subgenomic replicon, which provides the replication machinery of the virus and most non-structural proteins, and the coding RNA of all structural proteins and NS2 cloned from another isolate (Figure 2d). In theory, structural proteins and NS2 can be derived from any patient isolate. Unfortunately, genetic incompatibility between the various elements has been observed and heavily reduce efficacy of virus production. This phenomenon is mostly linked to non-structural proteins and particularly important when the genetic elements are derived from distinct genotypes [113]. This hurdle can be overcome, with some difficulty, by introducing specific adaptive mutations, deleting sequences in the subgenomic replicon dispensable for replication, and/or using custom-designed packaging systems for individual HCV genotypes [113, 125]. Efficient and easy production of HCV_{TCP} should contribute to studying HCV in entry and assembly steps of the HCV life cycles, in particular encapsidation of the viral genome.

3.2. Limitations of standard *in vitro* methods

All these methods use Huh-7 cells or derivative clones as substrate for HCV replication. These cells are by far the most permissive to HCV replication and, depending on mutations in genes involved in antiviral signaling, sustain high viral replication levels [126, 127]. Unfortunately, these cells are highly divergent from primary cells from which were derived, have different restriction mechanisms, lack of the cell polarity typically observed in hepatic tissue and primary hepatocytes, and do not exhibit same compartmentalization of HCV receptors. As a result, the mechanism of entry, assembly, release, and cell-to-cell spread observed *in vivo* is not completely reproduced *in vitro* [128].

Alternative cell substrates for HCV replication *in vitro* are HepG2 cell line and hepatoma cells. HepG2 derive from primary hepatocytes and polarize in culture. Unfortunately, HepG2 cells do not express CD81 and miR-122 - a microRNA highly expressed in the adult liver [129] and essential host factor for HCV replication (reviewed in chapter 4; [130]) - and are thus inherently resistant to HCV infection. To allow viral entry and replication, HepG2 cells have been engineered to express human CD81 and miR-122 [131, 132]. Hepatoma cells, in contrast, are CD81 and miR-122 positive and, depending on malignant and differentiation features, retain their ability to polarize in culture. Unfortunately, these cells are difficult to grow *in vitro* and often necessitate of specific medium supplements [133] and gel substrate in which to adhere [134]. On this regard, however, significant advancements have been obtained with hepatoma cell lines

capable of supporting HCV and hepatitis B virus replication and obtained from a liver cells of an HCV-infected patients that were further selected in nude mice [135].

HCV replication and persistence greatly depends on host factors and genetic background. It became clear in recent years, for instance, that host genetics influences disease progression, host immune response, and response to antiviral therapy [136, 137]). A small nucleotide polymorphism upstream of a λ-IFN gene, for instance, strongly correlates with disease and therapy outcome, and host-targeting antivirals (HTAs) have become an attracting field of drug research [138]. If hepatoma cell lines are the obvious choice, they do not fully recapitulate all aspects of HCV replication in the liver, let alone the extrahepatic target sites. Thus, HCVcc or equivalent systems based only on liver cells might provide partial and biased information on type and extent of host factors playing key roles in pathogenicity, and viral clearance or persistence.

3.3. Novel *in vitro* methods

Alternatives to the above-described methods are under investigation to achieve productive HCV infection in more physiologic conditions. Primary human hepatocytes from genetically diverse donors are difficult to obtain and can be propagated for short periods during which cells progressively loss differentiation. Human fetal liver cells, also difficult to achieve and exhibiting wide levels of permissiveness to HCV replication, can be cultivated longer and are generally more susceptible to HCV infection compared to adult human hepatocytes [139, 140].

In the last years the development of three-dimensional culture systems, natural and synthetic anchoring gels, co-culture methods, and cell reprogramming techniques have led to the establishment of novel cell substrates for HCV replication *in vitro*.

3.3.1. Semi-continuous cell lines of primary human hepatocytes

Co-cultivation with feeder cells prolong survival and have beneficial effects on biological functions of primary human hepatocytes [141]. Micropatterned co-cultures of primary human hepatocytes, usually seeded in small clusters and surrounded by fibroblasts, consent cell polarization and periods of cultivation compatible with HCV replication that however takes place at low efficiency and causes limited spread to contiguous cells due to innate immune response and other intrinsic resistant factors [142, 143]. To increase permissiveness, good results have been achieved by combining co-cultivation with strategies aimed to inhibit antiviral responses by engineering cells to express antiviral proteins that antagonize IFN-antagonists, treating cells with inhibitors of pathogen-associated pattern recognition receptors, reducing expression of genes involved in host defense by RNA interference, etc. [107, 144, 145].

3.3.2. Three-dimensional, tissue-engineered cultures

Compared to conventional cultures, three-dimensional cultures more accurately preserve multicellular architecture, viability, morphology, differentiation status, proliferation capacity, and gene expression profiles of cell populations. In virology, three-dimensional cultures are considered very important as they should maintain tissue-specific host factors, innate immune function, cell cycle status, and polarity resembling

analogous cells *in vivo* and thus better supporting the natural course of viral infection [141]. Another benefit of three-dimensional cultures is the possible combination of multiple cell types involved by viral infection *in vivo*. This approach has been used to demonstrate the dependence of hepatocyte function and growth on endothelial, Kupffer, fibroblasts, and other cells part of liver architecture [146, 147]. As described above, these interactions are also important in HCV infection, cell-to-cell transmission and, as demonstrated for Kupffer cells and liver sinuosoidal endothelium [148, 149], modulate HCV replication and disease progression. Successful attempts to create three-dimensional cultures of primary hepatocytes have been reported in recent years and are emergent *in vitro* models of studying hepatotropic viruses.

The models developed insofar use different scaffolding biomaterials and cell types and are supplemented with various growth factors or other biomolecules. Full description of technological platforms, advantages, and limitations is provided elsewhere [141, 150]. Three-dimensional cultures of hepatic tissue should enable the study of HCV infection in more physiological contexts, investigate virus spread in multiorgan models, and elucidate host-pathogen interactions in authentic host cells.

3.3.3. Production of hepatocyte-like cells from cell reprogramming

The development of induced pluripotent stem cells (iPSCs) [151] has shown a great potential in disease modeling, drug screening, organ reconstruction and cancer therapy. Human iPSCs offer the ability to produce host-specific differentiated cells and thus have the potential to transform the study of infectious disease. In 2011 Yoshida *et al* reported that iPSC-derived hepatocytes expressed HCV receptors, including CD81, SR-BI,

CLDN1, and OCLN; in contrast, the iPSC showed no expression of SR-BI or CLDN1. HCV RNA genome replication occurred in the iPSC-derived hepatocytes. Anti-CD81 antibody, an inhibitor of HCV entry, and interferon, an inhibitor of HCV genomic replication, dose-dependently attenuated pseudotyped HCV entry and HCV subgenomic replication in iPSC-derived hepatocytes, respectively [152].

Accordingly, in 2012 Schwartz *et al* [153] and Roelandt *et al* [154] reported that iPSC-derived hepatocytes, but not iPSCs, support the entire life cycle of HCV, including inflammatory responses to infection, enabling studies of how host genetics impact viral pathogenesis. Wu *et al* also reported that permissiveness to infection was correlated with induction of the liver-specific microRNA-122 and modulation of cellular factors that affect HCV replication [155].

A humanized mouse model consisting of triple knockout, combined immunodeficient (IL2R$\gamma^{-/-}$, Rag2$^{-/-}$) native hepatocyte-poor (fumarylacetoacetate hydrolase$^{-/-}$) mice reconstituted with human primary hepatocytes in the liver provides a novel experimental opportunity which mimics the *in vivo* growth of the human hepatocytes [156, 157]. Zhou *et al* introduced the personalized mouse model using human iPSCs (Figure 3) [158]. Accordingly, Carpentier *et al* reported that hepatocytes differentiated from hiPSCs could be engrafted in the liver parenchyma of immune-deficient transgenic mice carrying the urokinase-type plasminogen activator gene driven by the major urinary protein promoter. The hepatocytes were maintained for more than 3 months in the liver of chimeric mice, in which they underwent further maturation and proliferation. These engrafted and expanded human hepatic-like cells were permissive to

in vivo infection with HCV-positive sera and supported long-term infection of multiple HCV genotypes [159].

Recently, the field of HCV research has been revolutionized by the finding that pigtail macaque (*Macaca nemestrina*) hepatic cells derived from iPSCs support the entire HCV life cycle, and pigtail macaques may serve as a suitable, clinically relevant model for the study of HCV infection [160].

Moreover, the technologies generating vascularized and functional human liver from human iPSCs by transplantation of liver buds created *in vitro* have also been established. These findings will play a very important role in understanding the mechanism of HCV infection, spread in non-hepatic cells and tissues, and therapy for HCV related hepatocellular carcinoma [161].

3.3.4. In vitro models of extrahepatic HCV infection

Beside hepatocytes and as described in previous chapters, HCV RNA and proteins have been detected at low levels in lymphatic system, brain, gut, thyroid, etc. [49, 162-166]. Whereas several non-hepatic disease manifestations point to a direct role of HCV, it is not yet clear whether these tissues are truly infected (and potential extrahepatic reservoirs [167, 168]) or score positive due to infiltrating HCV-infected mononuclear cells. In this respect, an ever-growing body of literature suggests that blood cells are indeed an important site of HCV replication and persistence [39, 51, 52, 169]. It is unknown and object of intense research whether the antiviral compounds, tested in hepatic cells, possess same efficacy at blocking virus replication and curing infection.

Many attempts to establish an *in vitro* model for replication in non-hepatic cells have been attempted with controversial results. Several reports claimed successful replication of HCV_{cc} or HCV_{TCP} in B- and T-lymphocytes, monocytes/macrophages, and dendritic cells of healthy donors, in macrophages of HIV-infected patients, and cells of different histotype [33, 41, 170-176]). Unfortunately, HCV replication was detectable at low levels at best and all these models lacked consistency and reproducibility. The availability of an *in vitro* model for HCV replication using non-hepatic cells is therefore still an open issue. As further additional problem, there are no laboratory methods capable to firmly distinguish mere HCV detection from productive infection. Standard virological assays used for hepatoma cells are often inapplicable. Methods based on the detection of viral antigens are poorly sensitive to ascertain HCV infection [177]. In contrast, quantitative molecular assays that need to be highly sensitive for clinical diagnosis (see below) suffer from high background due to contamination by viral inocula or input RNA used for transfection. Various approaches, ranging from use of molecular clones with gene reporters, monitor expression of non-structural proteins, detection of replicative intermediate, etc., have been pursued to overcome this hurdle but there are no suitable *in vitro* method based on non-hepatic cells supporting robust HCV replication and exploitable for antiviral testing.

4. FINE-TUNING OF IN VITRO CULTURE METHODS AND MOLECULAR DIAGNOSIS TO MEET NEW PARADIGMS OF HCV THERAPY: AN EVER-CHANGING PROCESS

Availability of culture methods to replicate HCV *in vitro* prompted the study of virus and host factors as candidate targets for antiviral therapy. Structure and function of HCV protease and polymerase were predicted by molecular modeling before the advent of culture systems, but then again it was necessary an *in vitro* model of replication to assess efficacy of the approach and which lead compounds work best. The first proof of concept in the path of HCV DDAs came in 2003 with a small molecule from Bayer that inhibited the HCV protease *in vitro* and significantly reduced viremia *in vivo* [178]. In the same years the development of the replicon system represented a major breakthrough as it allowed direct testing of potential candidates to block HCV replication in a laboratory setting. The payback was substantial; there has been an explosion of new agents, targeting almost every phase of HCV replication and ranging from entry into the hepatocyte to assembly and virion release. Also thanks to the lessons learned with HIV, key targets of HCV therapy were found the HCV protease (NS3/4) and the polymerase (NS5 A/B).

As described in chapter 6, most clinically approved DDAs superseded the mainstay of IFN-RBV combined treatments to the point that IFN-free treatments are now available and the main goal of treatment has become cure, rather than halting disease progression. Such high stake has changed criteria and parameters defining response to therapy and residual viremia (see in the next paragraphs) and led to refocusing on use of *in vitro* systems. Up to few years ago, these methods were essentially used to test potency of HCV protease and polymerase inhibitors in hepatocytes. In the last years these methods where essentially used to investigate: 1. *Variability and mechanisms of resistance*. First *in vivo* trials clearly demonstrated that a single agent is not sufficient to produce sustained

viral suppression. Due to infidelity of viral RNA polymerases and high production (around 10^{12} virions per day [179], HCV rapidly accumulates an ensemble of viral variants, called quasispecies, each of those containing one or more mutations compared to parental virus. Using a single drug targeting a single block point will allow the rapid development of resistance. This led to the development of combinations of drugs targeting a different step of HCV replication steps or attacking the same viral molecule but having different sensitivities to mutations conferring resistance. Here, *in vitro* methods are essential to identify drug combinations that have synergistic effects and, usually following prolonged *in vitro* cultivation in the presence of suboptimal amounts of drug(s), identify viral pathways and mutations blunting the drug(s); *2. Antiviral efficacy across HCV genotypes.* Accumulation of mutations caused progressive divergence of viral variants intra- and inter-host that have been organized in genotypes and subgenotypes. Hierarchical classification is important for epidemiological properties and responsiveness to therapy. As discussed below, identification of infecting genotype (genotyping) is essential to define optimal IFN-RBV treatment and likelihood of clinical and virological responses. Structural differences among genotypes also impact current DDAs that have different levels of effectiveness against different block points, different regimes and combinations according to genotype. *In vitro* models are crucial to define the breadth of antiviral activity against genotypes (and subgenotypes) and to assist in the design of broad-spectrum therapeutic options; *3. Virus-receptor(s) interplay and development of entry inhibitors.* As described above, pathway and cellular actors involved in the entry process are only partly known. For most putative receptors, identified *in vitro*, it is not proved that they interact with the virus also *in vivo*, and work

the same way with all *in vitro* systems[180]. Identification of *bona fide* receptors and regions binding to E1 and/or E2 glycoproteins is essential to design molecules inhibiting viral entry. Since binding regions between viral and cellular receptors are usually highly conserved, the resulting entry inhibitor should be broad-range and possess a high genetic barrier, i.e. drug resistance occurs following acquisition of several critical mutations that usually heavily affects protein structure and function [181]; *4. Host target agents (HTAs).* Like any virus, HCV utilizes multiple host-encoded factors for cell entry, genome replication and virus assembly. In principle, all these factors are potential drug targets for HCV therapeutics, provided that blocking their function arrests HCV propagation without compromising their physiological activity or harming the cellular pathway to which they belong. *In vitro* studies have shown that this can be achieved with a number of HTAs that however need extensive testing in vitro and in suitable *in vivo* models before clinical approval comes into reach[138]. Maraviroc and similar drugs antagonizing chemokine receptors of HIV-1 provide proof of concept that safe and well-tolerated HTAs can be developed; *5. Antivirals and HTAs in extrahepatic cell target of HCV replication.* Even though DDAs in infected liver cells are extremely potent, there are no data for their efficacy in extrahepatic cells and tissues considered important site of HCV replication and persistence [33]. In this respect, the development of non-hepatic cell culture systems supporting HCV replication with which to test current DDAs and assist in the design of entry inhibitors or HTAs also effective in these cells will represent a great leap for HCV therapy; *6. Intracellular mechanisms of viral persistence and pathogenesis.* Since HCV does not integrate its viral genome during replication it is generally believed that upon successful and sufficiently long treatment the viral genome is degraded and the infected

tissue is eventually cured from infection. This fate has been postulated to occur and corroborated by the fact that most patients cleared the infection. It is not know whether this holds true in the long run, i.e. years after suspension of treatment. Ad hoc *in vitro* and *in vivo* experiments aimed to understand whether and how the virus persists in a non-replicative form intracellularly and if current therapy is effective are warranted. Similarly, the direct and indirect pathogenetic mechanisms leading to cell transformation and other ailments need to be elucidated and, albeit suggesting by numerous clinical trials as described in this thematic issue, no firm conclusions with *in vitro* studies have been reached. *7. Innate and genetic mechanisms of resistance.* Similarly to what observed with HIV, population studies of individuals at high risk for HCV infection suggest that protective mechanisms, whether immune and/or genetic, allowing clearance of infection without seroconversion do exist [182-184]. This evidence has been corroborated by *in vitro* studies showing that genetic polymorphisms influence susceptibility to infection and response to therapy [136]. *In vitro* culture systems to examine innate immunity and HCV interactome, and to manipulate genes associated with resistance to infection could identify potential targets for vaccine design and inform novel therapies.

As mentioned, efficacy of current HCV therapy has changed the requirements of clinically-approved molecular assays, and paradigms and clinical interpretation of the results achieved with molecular assays.

4.1. Molecular assays to diagnose HCV infection and response to therapy

Screening for HCV detection is carried out by examining blood samples with a combination of immunological and molecular assays. These latter have rapidly acquired a

pivotal role in the diagnosis of HCV infection and now are essential for the correct clinical management of infected patients, because they are used to quantitate the copies of viral RNA at baseline, during the course of treatment, and at the end of therapy [185, 186]. At least four ‘‘*in vitro* diagnostics’’ molecular platforms are currently available (Table 1). Although the intrinsic performances of these tests may differ, all use an automated RNA extraction followed by automated amplification and detection of HCV genome, and all are characterized by the same molecular principle based on real-time PCR technology. Currently, the assays demonstrate lower limits of detection range of 12-21 IU/ml across all HCV genotype and they are linear from 10 to 108 IU/ml with high accuracy and precision. Genetic variability of HCV is very high, thus correct selection of the viral genome segment targeted is critical for satisfactory performance of gene amplification assays. The 5’-untranslated region (UTR) is the most conserved portion of the genome and the more extensively used for HCV amplification even if it presents sufficient diversity between genotypes to affect assay sensitivity. The more recent versions of the assays have been redesigned to reduce this shortcoming, also with the use of a dual probe approach providing a broader detection and quantification of rare HCV sequences. It is important to note that, except for rare samples, currently the results of different assays are quite well comparable; however it is valuable that each patient is constantly monitored by using the same type of assay.

In the monitoring of HCV patients other molecular assays are used. Those for determining the infecting HCV genotype are essential because they help to decide type and duration of therapy as well as to predict treatment outcomes. Sequence analysis of the viral genome is considered the gold standard for genotyping HCV, and it also has

been shown that sequencing selected segments of the viral genome (i. e. E1, C, or NS5B regions) suffices for correct HCV genotype and subtype recognition [187, 188]. For practical purposes sequencing the 5′ UTR is considered acceptably accurate albeit not completely efficient at distinguishing some subtypes. Commercial kits for semiautomated sequencing are available, but alternative, easier-to-perform methods exist. A widely used commercial test exploits the ability of selected HCV amplicons to hybridize to type-specific probes immobilized on nitrocellulose strips (line probe assay). A recently updated version of this assay uses primers and probes that simultaneously target 5′ UTR and C regions improving identification of genotype 1 subtypes and accurately distinguishing genotype 1 from 6 [189].

4.2. Key considerations, interpretation of results and screening recommendation in the post-DAA era.

The goal of HCV therapy is a sustained virological response (SVR), defined as HCV undetectable in blood 24 weeks after the end of treatment. Early predictors of a future SVR have been investigated and a rapid virological response (RVR) characterized by “undetectable HCV RNA” (e.g < 50 IU/ml) after 4 weeks of standard PEG INF/RBV has been considered a good marker to shorten at 24 weeks (*vs* 48 weeks) therapy in genotype 1 infected patients. The recent development of DAAs and the approval of triple-therapy regimens have given new hopes for higher virus eradication rate in infected patients. That implies more accurate and sensitive quantitative HCV RNA tests for monitoring faster virus kinetics and promptly detecting treatment failures. These new drugs have revolutionized HCV treatment and they have led to the need of interpreting

more carefully the results of HCV RNA load (Tables 1 and 2). The concept of "undetectable HCV RNA" has been revised [186, 190]. Only a result defined as "Target not detected" at both 4 and 12 weeks of treatment is now believed discriminating for choosing patients eligible for shorten therapy being considered not more equivalent to an "detectable but below the lower limit of quantification (LLOQ)" HCV RNA result. Again, values above 25 IU/ml at 4, 12 or 24 weeks post-drug administration entail the stop of therapy while a "<25 IU/ml, HCV RNA detected" result is still considered acceptable for defining a patient with SVR. Not all commercial assays work similarly, in particular when very low amounts of HCV RNA have to be quantified. A study demonstrated that a large number of samples at 4 weeks of treatment resulted "Target not detected" when tested by a molecular assay but they have "detectable but below the LLOQ "HCV RNA levels with another commercial assay[191]. Then, depending to the HCV RNA assay used, the medical decisions on which patients have had to receive a shortened treatment regimen can be affected.

CONCLUSION

The development of methods that faithfully recapitulate in vitro the HCV life cycle has massively increased since the construction of the first replication-competent molecular clone from an isolate of an infected patient. Novel technologies such as three-dimensional cultures and cell reprogramming also promise to dramatically expand the possibility to study individual aspects of the HCV life cycle in primary hepatocytes, in vitro reconstructed liver tissue, or extrahepatic cells and tissues know to be target of HCV

infection in vivo. These novel substrates, together with HCVcc and HCV_{TCP} or similar systems permitting generation of inter-genotypic chimeras should allow studying some still obscure aspects of virus release and spread in a model more complicated but certainly closer to what takes place in vivo. Given the different in vivo relationships and outcomes of infection and disease exhibited by HCV, there is urgent need for in vitro systems permissive to patient-derived HCV isolates. The development of protocols to differentiate human and animal iPSC to fully functional hepatocytes and creation of mouse models engrafted with human iPSC-derived liver cells supporting HCV replication is certainly a big step toward this goal. Gene manipulation techniques with which to tweak hepatic cell genes permitting or restraining HCV replication combined with cell reprogramming and novel culture systems will offer the opportunity to finely dissect virus–host interactions as well as to understand the contribution of the host to resistance to infection or disease progression. Novel systems of HCV replication in vitro may help studying innate and immune mechanisms of cell resistance and assisting in the design of vaccine candidates. In more immediate terms, HCV culture systems already had important payoffs mainly consisting in the definition of targets for antiviral therapy, and development and testing of novel drugs so effective that in mere two years swiped away standard IFN-RBV therapy and revolutionized objectives and concepts of HCV therapy and patient management.

New concepts and goals of therapy also significantly impacted *in vitro* research and diagnostics. *In vitro* culture systems will be necessarily refocused toward the development of personalized medicine approaches using patient-derived HCV isolate to optimize the therapeutic regimen, analyze and characterize the emergence of resistance

mutations, and define the role of chemokines in the pathogenesis of HCV chronic infection and extrahepatic manifestations as well as markers of response to therapy [192-194].

More potent regimens with higher cure rates created complexity for laboratory diagnosis and clinicians. This has led to definition of new guidelines and interpretations of HCV RNA viral load defining SVR and response to therapy in general. Here, accurate quantitation, high sensitivity, and reproducibility and interchangeability of results among clinically-approved molecular platforms are even more important compared to what required with IFN treatment. Standardization of methods and equivalence of results is badly needed as well as definition of protocols to monitor adherence on-therapy, and early prediction of disease worsening and development of hepatocellular carcinoma.

Given the high cost of the new regimens and global burden of HCV chronic infection and disease in vitro culture systems and in vitro diagnosis will progressively increase their importance.

Conflict of interest

The authors declare that this article content has no conflicts of interest.

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1

Table 1. HCV RNA quantitative assays commercially available.

Assay	Manufacturer	Method (target region)	Lower limit of detection (IU/ml)	Lower limit of quantification (IU/ml)	Linear range of quantification (IU/ml)	In vitro diagnostic registration
Abbott RealTime HCV test	Abbott Molecular	Real-time PCR (5'-UTR)	12	12	12 to $1x10^8$	CE, FDA
Artus HCV QS RGQ kit	Qiagen	Real-time PCR (target proprietary)	21	35	35 to $1.77x10^7$	CE
COBAS AmpliPrep / COBAS TaqMan v2.0 test	Roche Molecular System	Real-time PCR (5'-UTR)	15	15	15 to $1x10^8$	CE, FDA
VERSANT HCV RNA 1.0 assay	Siemens	Real-time PCR (*pol* gene)	15	15	15 to $1x10^8$	CE

CE, European Community.

FDA, Food and Drug Administration.

2

3

Table 2. Specimen results and interpretations.

Reported result	Interpretation of the result
Target not Detected	No PCR amplification or detection, Ct value for HCV is above the limit for the assay or no Ct value for HCV is obtained
HCV RNA detected, less than the LLOQ IU/ml HCV RNA	Calculated IU/ml is below the lower limit of quantification (LLOQ) of the assay
"number" IU/ml, HCV RNA detected	Calculated result is quantifiable, greater than or equal to the LLOQ and less than or equal to upper limit of quantification (ULOQ)
"greater than the ULOQ IU/ml HCV RNA	Calculated result is above the linear range of the assay

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Figures

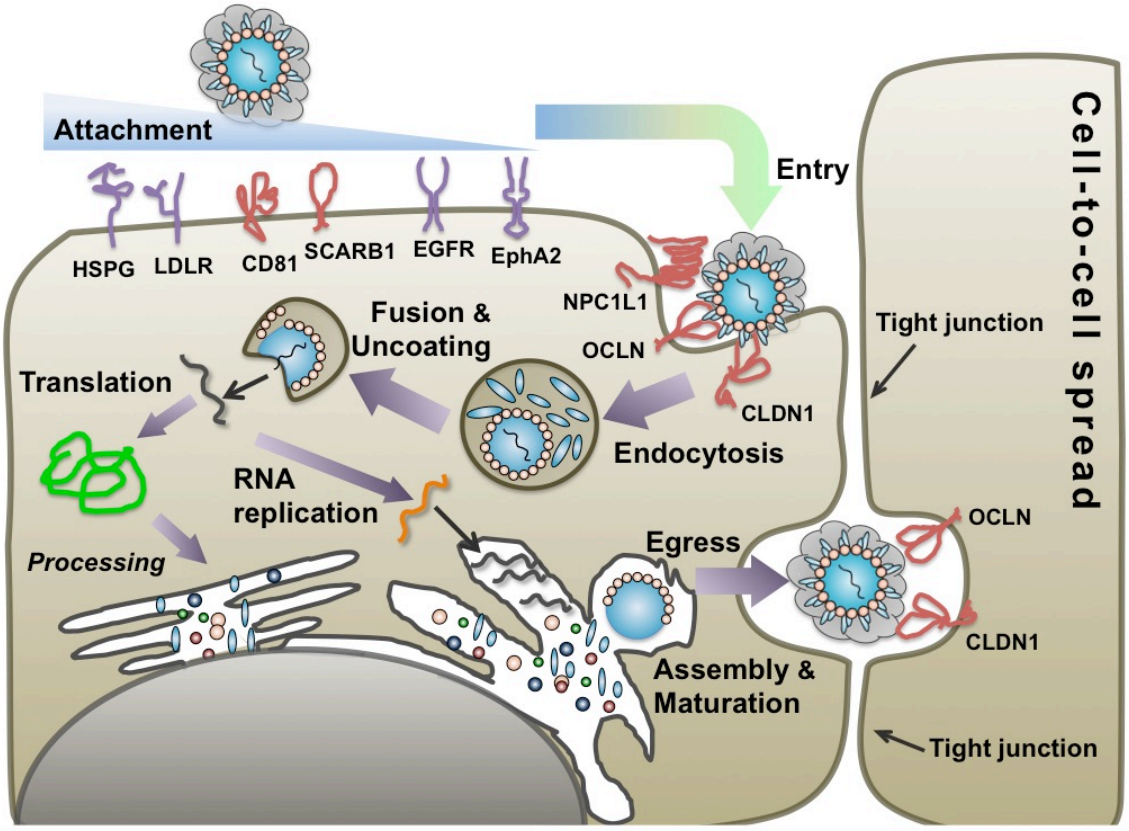

Fig. (1). Schematic of HCV life cycle and the five major steps of replication. The virus interacts with target cells by binding to receptors that, based on in vitro and in vivo entry inhibition tests with antibodies against the cellular molecules, are considered essential (shown in red) or accessory (purple) entry factors. Following attachment the virus is internalized through clathrin-mediated endocytosis, and the cellular and viral membranes are fused and capsid disorganized with a process triggered by the low pH of the endosome. After uncoating the positive-strand RNA genome is released into the cytoplasm and directly translated in a polyprotein precursor that is then cleaved into

single proteins by both host and viral proteases. The non-structural (NS) proteins and some host factors form a replication complex that synthesized multiple copies of the HCV RNA genome via a minus-strand replicative intermediate. Assembly of the nucleocapsid occurs into the endoplasmic reticulum from which the virion acquires the envelope with E1 and E2 glycoproteins. Maturation and association with endogenous lipoproteins to form lipoviral particles immediately follow. Virions are eventually released from the cells and transmitted to other cells via a cell-free mechanism or directly through tight junctions to neighboring cells by cell-to-cell spread. This process is likely mediated by only two receptors.

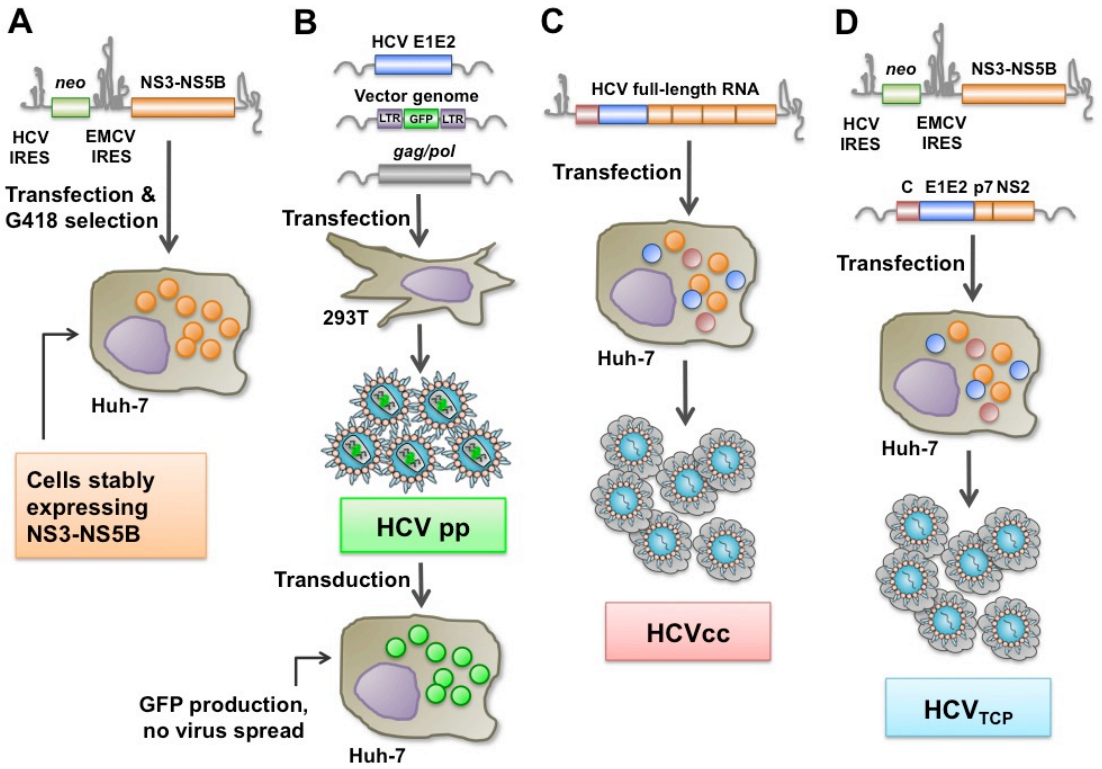

Fig. (2). Standard HCV cell culture systems. (**A**) The HCV replicon encodes the selection marker neomycin (*neo*) and the HCV replicase proteins (NS3-NS5B). The coding sequences are placed under control of the internal ribosome entry site (IRES) of HCV and encephalomyocarditis picornavirus (EMCV), respectively. Huh-7 cells are transfected with the HCV replicon and treated with G418 (an antibiotic) treatment, to select for cells harboring the HCV replicon and thereby expressing the antibiotic resistance gene (*neo*). (**B**) HCV pseudoparticles (HCVpp) are produced by co-transfection of human epithelial 293T cells with three expression vectors encoding HCV E1 and E2, HIV or murine leukemia virus (MLV) Gag-Pol, and HIV or MLV genome encoding a reporter gene (typically luciferase or green fluorescent protein [GFP]). HCVpp produced and released by 293T cells are collected and used to evaluate/quantitate

HCV entry into Huh-7 or other target cells. (**C**) Cell-culture derived HCV (HCVcc) is generated by transfecting Huh-7 with a full-length HCV RNA genome. The released virions are replication competent and associated with lipoproteins as for the virus produced in vivo. (D) Trans-complemented HCV particles (HCV_{TCP}), are obtained by co-transfection of Huh-7 with the HCV replicon shown in Fig. 1A and an expression vector encoding the structural and NS genes missing in the HCV replicon and necessary to produce HCV virions. These particles are associated with lipoproteins but incapable to replicate. This system is used to overcome the hurdle of isolate specificity and provide isolate-specific information for HCV entry, replication and assembly. Typically uses the subgenomic replicon from the replication-competent molecular clone of JFH1 and missing genes derived from a specific isolate.

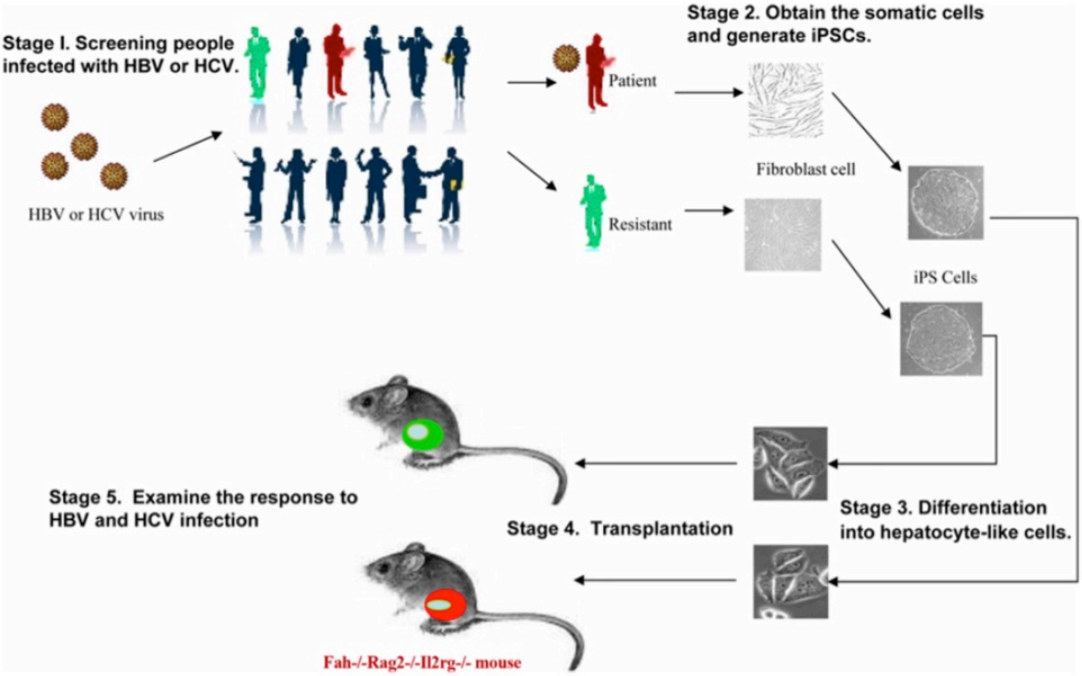

Fig. (3). Strategies for developing a personalized mouse model for hepatitis B virus and HCV using personalized human induced pluripotent stem (iPS) cells (adapted from ref [158], with permission from the Author).